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Ex-vivo expanded human blood-derived CD133$^+$ cells promote repair of injured spinal cord

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ABSTRACT

Human blood-derived CD133$^+$ cell populations, which are believed to represent a hematopoietic/endothelial progenitor fraction, have the ability to promote the repair of injured spinal cord in animal models. However, the mechanisms by which CD133$^+$ cell transplantation promotes spinal cord regeneration remain to be clarified. Another possible hurdle on the way to clinical applicability of these cells is their scarce representation in the overall population of mononuclear cells. We therefore analyzed and compared ex-vivo expanded human cord blood derived CD133$^+$ cells with freshly isolated CD133$^+$ cells as well as corresponding CD133$^-$ control mononuclear cells in respect to their ability to promote spinal cord repair using in vitro assays and cell transplantation into a mouse spinal cord injury model. In vitro, expanded cells as well as fresh CD133$^+$ cells formed endothelial progenitor cell (EPC) colonies, whereas CD133$^-$ cells formed no EPC colonies. In vivo, the administration of fresh CD133$^+$ and expanded cells enhanced angiogenesis, astrogliosis, axon growth and functional recovery after injury. In contrast, the administration of CD133$^-$ cells failed to promote axon growth and functional recovery, but moderately enhanced angiogenesis and astrogliosis. In addition, high-dose administration of expanded cells was highly effective in the induction of regenerative processes at the injured spinal cord.

1. Introduction

Spinal cord injury (SCI) is one of the major causes of serious paralysis, and is intractable as the central nervous system (CNS) has a limited capacity to regenerate. Although several cell-based therapies for SCI have been reported in recent years, no optimal cell source has yet been determined [1–4]. The population of CD133$^+$ cells in human blood, known to be a subpopulation of CD34$^+$ cells, is well characterized as a hematopoietic/endothelial progenitor fraction [5–10]. Previous studies reported that the transplantation of human circulating CD34$^+$ cells was effective in enhancing the repair of the injured CNS [11,12]. In addition, our previous studies showed that the transplantation of human blood-derived CD133$^+$ cells promoted functional recovery after spinal cord injury [13,14]. However, the mechanisms for spinal cord repair promoted by human CD133$^+$ cell transplantation still remain elusive. CD133$^+$ cells in human blood appear to be suited for clinical application as isolation of these cells is relatively safe and raises only limited ethical concerns. However, the acquisition of a sufficient number of cells remains the most important factor limiting clinical application, as the proportion of CD133$^+$ cells in respect to the overall number of mononuclear cells (MNCs) in human blood is very small [8]. We developed a serum-free quality and quantity (QQ) control culture system for human cord blood derived CD133$^+$ cells enhancing the cellular function as well as number of endothelial progenitor cells destined to be used for the revascularization of ischemic organs and tissues [15]. The purpose of this study was to assess the capacity and potential of expanded CD133$^+$ cells to induce or enhance the repair of injured spinal cord, and to clarify the mechanisms underlying the transplantation of these cells based regenerative events.

2. Materials and methods

The Institutional Animal Care and Use Committees of RIKEN Center for Developmental Biology approved all animal procedures in this

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study. The use of human cord blood for this study was also approved by the ethical committee of the RIKEN Center for Developmental Biology.

2.1. Isolation and expansion of CD133 positive cells from human cord blood

Human umbilical cord blood was supplied by Aywill, Inc., Kobe, Japan. $CD133^+$ cells were isolated from human cord blood as reported previously [16]. Total MNCs were isolated by density gradient centrifugation from cord blood. $CD133^+$ cells were separated from MNCs using CD133-bound microbeads and a magnetically activated cell sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturer's protocol. Subsequently, $CD133^+$ and $CD133^-$ fractions were collected. Approximately half of the $CD133^+$ cells were expanded by a QQ culture [15]. The harvested $CD133^+$ cells were cultured with serum-free medium (StemSpan™; StemCell Technologies, Vancouver, BC, Canada) supplemented with 100 ng/ml stem cell factor (SCF), 50 ng/ml vascular endothelial growth factor (VEGF), 20 ng/ml interleukin-6 (IL-6), 20 ng/ml thrombopoietin (TPO), 100 ng/ml fms-like tyrosine kinase-3 ligand (Flt-3L) for 7 days. In order to analyze and confirm the purity of the $CD133^+/CD34^+$ cells in the freshly isolated $CD133^+$ cells and the one week expanded cells, regular fluorescence-activated cell sorting (FACS) analysis was carried out with a FACS Calibur analyzer (Becton Dickinson, San Jose, CA) and CellQuest™ Pro software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Cells were stained with phycoerythrin (PE)-conjugated anti-CD34 (clone 581, BD Pharmingen, San Diego, CA) and allophycocyanin (APC)-conjugated anti-CD133 antibodies (clone 293C3, Miltenyi Biotec). After washing, the cells were resuspended in staining medium supplemented with 7-aminoactinomycin D (7-AAD). Stained cells were analyzed by FACS, and dead cells stained with 7-AAD were excluded from analysis.

2.2. EPC colony forming assay

An endothelial progenitor cell (EPC) colony-forming assay was performed, as reported previously [17]. The number of EPC colonies was assessed after culturing 500 $CD133^-$ MNCs, fresh $CD133^+$ cells or expanded cells for 12 days in methyl cellulose-containing medium M3236 (StemCell Technologies) with 20 ng/ml SCF, 50 ng/ml VEGF, 20 ng/ml IL-3, 50 ng/ml basic fibroblast growth factor (bFGF), 50 ng/ml epidermal growth factor (EGF), 50 ng/ml insulin-like growth factor-1 (IGF-1) and 2 U/ml heparin. Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Scheffe's post hoc comparisons. In addition, the endothelial characters of the EPC colonies were confirmed by the cytochemical positivity for fluorescein conjugated *Ulex europaeus* agglutinin I (UEA-I; Vector Laboratories, Burlingame, CA) and immunoreactivity for endothelial nitroxide synthase (eNOS; Sigma, St. Louis, MO).

2.3. Real-time PCR analysis of $CD133^-$ MNCs, $CD133^+$ cells and expanded cells

Total RNA was obtained from human cord blood $CD133^-$ MNCs, $CD133^+$ cells, and expanded cells ($n = 8$ in each group) using RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan) according to the manufacturer's instructions. After first-strand cDNA synthesis via the PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Japan), real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with ABI Prism 7700 (Applied Biosystems, Foster City, CA) using SYBR Green Master Mix reagent (Applied Biosystems) according to the manufacturer's protocol. The relative mRNA expression was calculated for each gene by the 2-ΔΔCT method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization [18]. Results were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Scheffe's post hoc comparisons. Each primer sequence is shown in Table 1.

2.4. Spinal cord injury model

All surgical procedures were performed using an operating microscope (Zeiss, Oberkochen, Germany). Female nude mice (BALB/c-nu/nu, 12 weeks old, weighing 22–24 g) were anesthetized with an intraperitoneal injection of 400 mg/kg 2,2,2-tribromoethanol (Avertin; Sigma). After a laminectomy at the 10th thoracic spinal vertebrae, we exposed the dura mater. Spinal cord crush injury was performed by compressing the cord laterally from both sides with number 5 Dumont forceps (Fine Science Tools, North Vancouver, BC, Canada) for 10 s as previously reported [19]. The bladders of the treated mice were emptied manually once a day until restoration of autonomic bladder function.

2.5. Cell transplantation

Cell transplantation was performed immediately after spinal cord injury. 5×10^4 of $CD133^-$ MNCs, fresh $CD133^+$ cells or expanded cells were suspended in 200 μl of phosphate buffered saline (PBS) and injected intravenously (CD133-group, Fresh group and Expanded group). 1×10^6 of expanded cells were administered into mice of the high-dose transplantation group (Expanded-H group). 200 μl of cell free PBS was injected into control mice (PBS group).

2.6. Behavioral testing

The recovery of hindlimb motor function was assessed using the Basso Mouse scale (BMS) [20]. The mice in each group ($n = 8$) were assessed before SCI and 1, 4, 7, 14, 21and 28 days after injury and evaluated in an open field by two observers blind to the experimental conditions. Results were expressed as mean ± standard error. Statistical analysis was performed using two-way repeated measures ANOVA for group × time and Scheffe's post hoc comparisons.

2.7. Electrophysiological recording

Signal conduction in the motor pathway was assessed by the measurement of motor evoked potentials (MEPs) at 4 weeks after injury as described previously [19]. Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride, which has little effect on MEPs [21]. Mice were fixed in a stereotaxic apparatus. A pair of needle electrodes was placed subcutaneously at 3 mm on each side of the vertex of the skull. The motor cortex was stimulated transcranially with 0.2 ms square wave pulses using a constant current of 50 mA. Electromuscular responses were recorded from both hamstring muscles using a commercially available system (Viking

Table 1
Primers for real-time PCR (human).

Gene	Forward primer	Reverse primer
VEGF-A	CTGCCTCCTGACACTTCCTC	TTTCTTGCGCTTTCGTTTTT
VEGF-B	CATACCAGCTCAGGGGAGAA	CCCATTATGCAGAGGTTTGG
VEGF-C	TTGTGGGTCCATGCTAACAA	TGTTTGGTCATTGGCAGAAA
VEGF-D	TCCCTGGTTCATTGATGGAT	ATTACATGGGTCCCCTCCTC
PlGF	GCCTGGATGAGAAACAGCTC	GAGAATCTGGCTTGGCAGTC
Ang1	GAAGGGAACCGAGCCTATTC	AGGGCACATTTGCACATACA
Ang2	ATGCTAACAGGAGGCTGGTG	CCTTGAGCGAATAGCCTGAG
IGF1	TGCAGGAGGGACTCTGAAAC	ACATGGTATTTGGGGCCTTT
IGF2	CGTTTCCATCAGGTTCCATC	TGAAGATGCTGCTGTGCTTC
BDNF	GTCCCTGTATCAAAAGGCCA	CTTATGAATCGCCAGCCAAT
HGF	TGGATGCACAATTCCTGAAA	TGATCCAATCTTTTCAGCCC
EGFL7	AGGAAGAAGTGCAGAGGCTG	GCTCCTCCAGGAAGGAAATC
TSP1	TTGTCTTTGGAACCACACCA	CTGGACAGCTCATCACAGGA
IGFBP4	AGGGAGGTGGGGTACATTTC	AATGGGGTAGGGAACAGGAC
IGFBP7	AAGTAACTGGCTGGGTGCTG	TATAGCTCGGCACCTTCACC
TNFα	AGCCCATGTTGTAGCAAACC	TGAGGTACAGGCCCTCTGAT
IL6	AGTTCCTGCAGAAAAAGGCA	GAGGTGCCCATGCTACATTT
IL8	CAGGAATTGAATGGGTTTGC	AAACCAAGGCACAGTGGAAC
GAPDH	TTCTAGACGGCAGGTCAGGT	ACCCAGAAGACTGTGGATGG